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EFFECTS OF BENZENE VAPOUR ON THE γ-AMINOBUTYRIC ACID (GABA) SYSTEM IN RAT BRAIN

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Abstract—Chronic benzene intoxication (0·35 mg/l.) initially caused an increased in GABA concentrations with a simultaneous decrease in the glutamic and aspartic acid levels in cerebellum and pons Varolii of rats. On the 14th day of chronic exposure the level of all three amino acid was increased and associated with a depressive character of EEG patterns throughout the cerebellum, pons Varolii, and the sensori-motorial region of the cerebral hemispheres. On the 30th day, the GABA level had almost returned to its initial level but concentrations of glutamic and aspartic acid were decreased by more than 60% in cerebellum and by 20% in pons Varolii. During acute benzene poisoning (35·0 mg/l, for 15 min) the level of GABA was increased 5–6 times above the normal values. A 45% increase in the GABA level and a decreased amount of glutamic and aspartic acid were still observed 48 hr after the cessation of benzene inhalation. GAD activity in cerebellum and pons Varolii, during acute and chronic benzene intoxication, was higher than that of untreated animals: it was greater on the 14th day of chronic intoxication, 5- and 2-fold, respectively, and 4- and 3-fold at the 15th minute of acute intoxication. The GABA-T activity was also increased in these two structures (4–5-fold) by low and high concentrations of benzene vapour.

Functional disorders of the central nervous system are the early symptoms of acute and chronic benzene intoxication which precede the development of hematological alterations [1, 2]. Chronic benzene poisoning produces changes in the clinical status of the internal organs, circulating formed elements of the blood, and blood pressure [3, 4]. Benzene intoxication causes also a disturbance of the coordination of movements and muscular exercises connected with nerve cell activity of the cerebellar and pontic regions [2, 4]. In this connection, the determination of indices of permissible benzene concentrations (atmospheric pollution) is important for the health and safety of petrochemical workers.

The purpose of the present investigation was to measure the effect of low and high concentrations of benzene vapour on the GABA content of the brain (cerebellum, pons Varolii) and on the activities of the enzymes involved in the synthesis and destruction of this amino acid (L-glutamate carboxylase GAD, EC 4.1.1.15 and 4-aminobutyrate: 2-oxoglutarate aminotransferase, GABA-T, EC 2.6.1.19). This investigation of components of the GABA system, i.e. of an inhibitory neurotransmitter [5–11], would contribute to understanding the interrelation between neurochemical reactions of GABA metabolism and functional changes of the central nervous system state during benzene intoxication. No published data concerning the level of GABA and activities of enzymes controlling its metabolism in brain under benzene intoxication appear to exist in the literature.

METHODS

All experiments were performed with albino male rats weighing approximately 250–300 g. The animals were killed by decapitation and the brains were dissected in the cold as quickly as possible. The brain tissue was prepared according to our modification [12] of the method of Roberts [13]. The tissue samples (cerebellum, pons Varolii) were homogenized in 10 vol, ice-cold, 75% ethanol. The precipitated proteins were separated by two centrifugations. The combined supernatant fluids were evaporated to dryness. The residue was dissolved in 3·5 ml water, was centrifuged, and the aqueous supernatant was evaporated to dryness. The amino acids (GABA, glutamic and aspartic acids) were separated by paper electrophoresis using water–acetic acid–pyridine (44:8:1, by vol) (pH 3·5) as a buffer. Electrophoresis was carried out at room temperature. The electric field had a voltage drop of 30 V/cm in 2 hr. The electrophorograms were dipped into 0·5% ninhydrin solution in acetone and subsequently heated at 70° in a drying cabinet for 10–15 min. The areas carrying the amino acid spots were cut and eluted with 0·005% $CuSO_4$ in 75% ethanol. All readings were made with electrophotocolorimeter model FEK-M at 530 nm.

Determination of brain glutamic decarboxylase [14]. The activity of GAD in brain homogenates was determined by measuring the increase in GABA during incubation of brain homogenates with glutamic acid. The animals were killed by decapitation. The tissue samples were homogenized immediately in two vol of ice-cold 0·05 M phosphate buffer, pH 6·3, using glass homogenizers at 4°. The incubation mixture contained 1·0 ml of the brain homogenate and 10 ml of 0·05 M glutamic acid (neutralized to pH 6·3–6·7). Samples were incubated in closed tubes for 30 min at 37° under anaerobic conditions (N_2). At the end of the incubation the reaction was stopped by heating for 10 min at 100°. Five ml of water was then added, and after shaking, the samples were centrifuged. The

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control samples were heated immediately before incubation. The samples were assayed for GABA by a common procedure based upon paper electrophoresis. Activity is expressed as μmoles of GABA formed per g fresh tissue per hr.

Determination of brain γ-aminobutyric acid-α-ketoglutaric acid transaminase activity. The brains of decapitated rats were homogenized with two vol ice-cold 0·05 M phosphate buffer, pH 7·8. The incubation mixture contained 1 ml of the brain homogenate, 0·5 ml of α-ketoglutaric acid (0·05 M in pH 7·8–8·2 phosphate buffer) and 0·5 ml of GABA (0·05 M in pH 7·8–8·2 phosphate buffer). The incubation procedure was identical to that described for the determination of brain GAD activity. Activity is expressed as μmoles of glutamic acid formed per g of fresh tissue per hr.

All data obtained were examined statistically by the *t*-test [15].

Inhalation chamber (volume 100 l. Before carrying out experiments, the animals, during a control period, were placed every day in the inhalation chamber (but without exposure to benzene). For the study of the influence of high concentrations (35 mg/l.) of benzene vapour, 3·5 ml of chemically pure benzene, with a specific gravity of 0·879, was injected into the chamber. Every 55 min the chamber was ventilated for 5 min for an even distribution of benzene vapour. At this concentration (35 mg/l.), in 3·5 hr symptoms of poisoning, i.e. trembling, rigidity of the tail, lateral position, etc., were observed. To study the influence of such a concentration of benzene vapour during 3·5-hr exposures, animals were killed after intervals of 5, 15, 30, 60 min and 3·5 hr after the beginning of inhalation. To study the chronic influence of a low concentration (0·35 mg/l.) of benzene vapour, the animals were placed in the inhalation chamber for 5·5 hr every day for 1 month. Benzene was injected into the chamber each time to produce a vapour concentration of 0·35 mg/l. The animals were killed for analyses on days 1,7,14 and 21 after the beginning of daily, chronic inhalation.

Recording of electroencephalographic (EEG) activity. Chronic unipolar electrodes were implanted in brain structures of rats, including sensori-motorial regions of the cerebral hemispheres, the cerebellum, and the pons Varolii using a stereotaxic apparatus (type STM-GN, U.S.S.R.) intended for small laboratory animals. The electrodes were nichrome wire (250–300 μm dia) with a factory varnished insulation. Tentative location of these electrodes was determined by X-radiography. Histological verification of electrode placements (haematoxylin–eosin stained sections) indicated that such nervous structures as cerebellum, pons Varolii and sensori-motorial cortex were, in fact, being recorded. EEG recordings during benzene intoxication were made using an 8-channel ink-writing electroencephalograph (Model 0/3, Medicor Mfg., Hungary).

Histological investigation. The brain structures (cerebellum and pons Varolii) were fixed in 10% formalin. The sections were stained by haematoxylin–eosin.

RESULTS

Effect of benzene intoxication on the GABA system. Table 1 shows the levels of GABA, glutamic, and aspartic acids in the cerebellum and pons Varolii during chronic benzene intoxication (0·35 mg/l. for 5·5 hr daily for up to 30 days). At the 7th day of this chronic benzene inhalation, the GABA content of cerebellum was increased almost 2-fold and was 33% higher than that of control in the pons Varolii. The levels of glutamic and aspartic acids were decreased in both brain structures. At the 14th day there was a maximal increase of these three amino acid levels and a decrease at the 21st day of the chronic benzene vapour treatment. By the 30th day the GABA level corresponded to that of the 7th day of intoxication. The concentrations of glutamic and aspartic acids continued to decrease during this period: their level was more than 66% decreased from normal values in cerebellum and was decreased by 20% in the pons Varolii. Data in Table 2 shows the alteration of amino acid content during high concentration (35 mg/l.) benzene intoxication. The changes in the brain GABA (cerebellum, pons Varolii) within 5 min after benzene inhalation were significant: its level was 4-fold greater than that in the brain tissue of control animals. After 15 min, the GABA concentration reached its maximal level 5–6 times above that in the brain of control animals. There was a simultaneous increase in the glutamic acid levels in the brain structures of poisoned animals. The content of aspartic acid was higher only in the pons Varolii at the 15th min of acute intoxication. Then after 30 min the GABA level decreased 59–71% with the simultaneous diminution of other amino acids. After 1 hr the glutamic acid content was decreased by 22% in comparison with its control value. After 3·5 hr the GABA amount continued to decrease but did not reach its initial level. In cerebellum and pons Varolii the GABA concentration was 2·5-fold greater than the control values. At the same time the levels of aspartic acid decreased by 20–22%, but the 40% decrease of the glutamic acid content was found only in cerebellum. The increase of 45% in the GABA level and the decreased concentration of other amino acids were still apparent 48 hr after the removal of the animals from the inhalation chamber.

The GAD activity of these brain structures during benzene intoxication (at both vapour concentrations) was higher in cerebellum and pons Varolii on the 30th day of chronic intoxication and 3·5 hr after acute intoxication. The GABA-T activity was also increased in these two structures: it was 2-fold higher in the benzene intoxicated animals from the group inhaling the low concentration (0·35 mg/l.; on the 30th day) and 3 times greater in the group exposed to high concentration (35·0 mg/l.; 3·5 hr after acute intoxication) of benzene vapour (Table 3). The appearance of the GABA peak on the 14th day of chronic intoxication and at the 15th minute of acute intoxication corresponds to the increased activity of its enzymes. The GAD activity was higher on the 14th day of benzene intoxication in cerebellum and pons Varolii, 5 and 2-fold, respectively, and 4 and 3-fold at the 15th minute of acute intoxication. The GABA-T activity was also increased in cerebellum and pons Varolii: it was 4·5 and 3·5-fold higher respectively, on the 14th day and 5 (cerebellum) and 4 (pons Varolii) times greater at the 15th minute of acute intoxication.

Histopathological changes in nervous cells of cerebellum and pons Varolii. The short-term and prolonged

Table 1. Content (μmoles/g wet wt) of GABA, glutamic and aspartic acids in cerebellum and pons Varolii of rats at various stages of chronic benzene vapour intoxication (0·35 mg/l.)

Stages	Cerebellum GABA	P	Glutamic acid	P	Aspartic acid	P	pons Varolii GABA	P	Glutamic acid	P	Aspartic acid	P
Control	1·05 ± 0·13	—	5·51 ± 0·5	—	4·39 ± 0·36	—	1·23 ± 0·07	—	2·98 ± 0·06	—	1·57 ± 0·1	—
7 days	1·91 ± 0·12	<0·01	3·94 ± 0·39	<0·02	2·82 ± 0·1	<0·01	1·64 ± 0·1	<0·01	2·48 ± 0·06	<0·01	1·27 ± 0·07	<0·02
14 days	6·97 ± 0·58	<0·001	7·0 ± 0·28	<0·02	4·33 ± 0·44	>0·5	5·77 ± 0·11	<0·001	4·49 ± 0·15	<0·001	2·46 ± 0·14	<0·01
21 days	2·51 ± 0·23	<0·01	4·07 ± 0·32	<0·02	2·87 ± 0·37	<0·05	3·02 ± 0·15	<0·001	2·46 ± 0·1	<0·01	1·17 ± 0·05	<0·02
30 days	1·87 ± 0·13	<0·01	2·31 ± 0·65	<0·001	1·91 ± 0·27	<0·001	1·64 ± 0·1	<0·019	2·42 ± 0·07	<0·01	1·21 ± 0·06	<0·02

Results are means ± S.E.M. of ten experiments.

Table 2. Content (μmole/g wet wt) of GABA, glutamic and aspartic acids in cerebellum and pons Varolii of rats at various stages of acute benzene vapour intoxication (35·0 mg/l. for 15 min)

Stages	Cerebellum GABA	P	Glutamic acid	P	Aspartic acid	P	pons Varolii GABA	P	Glutamic acid	P	Aspartic acid	P
Control	1·05 ± 0·13	—	5·51 ± 0·15	—	4·01 ± 0·36	—	1·28 ± 0·07	—	2·98 ± 0·06	—	1·57 ± 0·1	—
5 min	4·31 ± 0·25	<0·001	6·94 ± 0·37	<0·05	4·39 ± 0·11	>0·5	4·40 ± 0·28	<0·01	5·41 ± 0·32	<0·01	3·26 ± 0·22	<0·01
15 min	6·35 ± 0·27	<0·001	6·75 ± 0·16	<0·05	4·35 ± 0·37	>0·5	7·76 ± 0·09	<0·001	6·57 ± 0·36	<0·001	3·97 ± 0·16	<0·001
30 min	2·61 ± 0·32	<0·01	5·32 ± 0·23	>0·5	4·28 ± 0·37	>0·5	2·23 ± 0·17	<0·05	2·52 ± 0·3	>0·2	1·42 ± 0·44	>0·1
60 min	3·68 ± 0·37	<0·01	4·49 ± 0·22	<0·01	4·54 ± 0·26	>0·2	3·85 ± 0·17	<0·001	1·97 ± 0·2	<0·2	1·08 ± 0·11	<0·05
3·5 hr	2·50 ± 0·16	<0·01	3·29 ± 0·67	<0·01	3·14 ± 0·34	<0·05	2·83 ± 0·05	<0·001	2·46 ± 0·17	>0·2	1·21 ± 0·05	<0·05
48 hr after cessation of benzene inhalation	1·78 ± 0·13	<0·01	2·69 ± 0·33	<0·001	2·56 ± 0·21	<0·001	2·07 ± 0·06	<0·001	2·59 ± 0·04	>0·2	1·26 ± 0·07	<0·05

Results are means ± S.E.M. of ten experiments.

Table 3. Effect of benzene intoxication on the activity of GABA system enzymes (GAD and GABA-T) in cerebellum and pons Varolii of rats

Brain Structure	GAD activity (μmole GABA/g per hr) benzene (mg/l.)					GABA-T activity (μmole GA*/g per hr) benzene (mg/l.)				
		0·35†		35·0‡			0·35†		35·0‡	
	Normal	14 days	30 days	15 min	3·5 hr	Normal	14 days	30 days	15 min	3·5 hr
Cerebellum	21·5 ± 0·58	119·6 ± 1·15	64·2 ± 0·92	95·6 ± 1·73	70·2 ± 0·87	24·4 ± 0·98	139·0 ± 2·02	55·6 ± 0·86	126·8 ± 1·09	91·0 ± 2·2
Pons Varolii	41·1 ± 0·53	82·0 ± 1·2	75·9 ± 1·57	129·7 ± 1·19	84·6 ± 0·78	29·6 ± 1·27	103·4 ± 1·67	55·0 ± 2·33	119·2 ± 1·56	114·6 ± 4·54

* GA, glutamic acid.
† Animals were killed by decapitation after 14 and 30 days of chronic intoxication.
‡ Animals were killed by decapitation after 15 min and 3·5 hr of acute intoxication.
Results are mean ± S.E.M. of ten experiments.

inhalations of benzene vapour at two concentrations caused appreciable destructive changes of nerve cells. There were different degrees of ganglionic cell dystrophy: solution of tigroid masses, focal hemorrhages, swelling of blood vessel endothelium, and reactive growth of ependymal cells. Different stages of dystrophic changes were found in Purkinje cells, which were enucleated and denuded of cellular processes during the benzene intoxication period.

DISCUSSION

The low concentration (0·35 mg/l.) of benzene vapour increased the GABA level in cerebellum and pons Varolii, and simultaneously decreased the concentration of glutamic acid with increased GAD activity. The greatest augmentation of the GABA level at the 14th day of chronic benzene intoxication corresponded to a depressive character in EEG records which showed pronounced changes in spike activity of the cerebellum and pons Varolii. It is possible that accumulation of GABA in nerve cells of the cerebellum indicates a protective reaction of the organism against the benzene action. After 30 days of chronic intoxication the functional state of the central nervous system is a general 'inhibition' (shown by the depressive character of bioelectric activity of cerebellum) with a background of an increased GABA level. The sharp decrease of the glutamic acid concentrations can, perhaps, be explained by inhibition of brain metabolic reactions. Symptoms of acute intoxication were distinctly shown after exposure to high concentrations (35·0 mg/l., for 3·5 hr) of benzene vapour, the GABA level increasing 4–6 fold during the first 3–15 min. In animals that survived the stage of acute intoxication, the brain GABA concentration decreased, but remained about 80% higher than that of controls, although the levels of glutamic and aspartic acids were at their lowest. Therefore, these indices show similarities between benzene intoxidation at low (during 1 month) and high (in 48 hr) concentrations. Analysis of EEG activity shows a slight tendency towards normalization of bioelectrical activity 48 hr after inhalation of benzene at a high concentration. In the case of chronic action of low concentrations of benzene (0·35 mg/l. during 30 days) the spikes of electrical output of cerebellum remained altered with appreciable depression till the end of the observaton, in comparison with pretreatment or control records.

The 'toxicity' of benzene is the sum of its interactions with nervous cell constituents to produce chemical alteration plus the cell's response to these aberrations. At present it is difficult to explain the increased activity of GABA metabolizing enzymes in nervous structures of cerebellum and pons Varolii under action of benzene vapour of various concentrations. The enhancement of the GABA level in cerebellum in our experiments was accompanied by a large decrease of glutamate and increased GAD activity. In the case of a disturbance of important and vital systems of the whole organism, beginning with disturbances in the blood circulation and respiration, one expects some manifestation of the compensatory, protective possibilities inherrent in the organism. Perhaps the increased GABA level can provoke a deep inhibition of nerve cells which contributes to their preservation. It is possible that the normal chemical reactions among the nervous system's metabolism lead to the detoxification of the toxic benzene doses. The combination of glycine with free phenolic acids, for instance, eliminates their inhibitory action of GAD and even favors the intensification of its enzymatic activity [16]. The increased GABA-T activity must also promote a sharp rise in GABA utilization with a resultant decrease in its concentration to normal or even lower levels. The transamination of GABA with α-ketoglutaric acid proceeds without energy-rich phosphate bond synthesis, which would allow the oxidative processes in nerve cells to lose, to some extent, their dependence on a fixed concentration level of inorganic phosphate.

Normal homeostatic processes appear to maintain the brain GABA content at a stable level that indicates the great metabolic plasticity of the nervous system. Thus, GABA content in brain is not always connected with the degrees of enzyme activity related to GABA metabolism but depends also on many other factors [9, 14, 17–19]. However, it should be acknowledged that GABA is continuously formed in neuronal cells and has a modulatory influence on the central nervous system during different functional states. Elucidation of fine molecular mechanisms of regulating neurochemical processes in response to benzene intoxication will be a subject of our subsequent investigations.

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